

Rap signaling is crucial for the competence of IL-7 response and the development of B-lineage cells

Yoshinori Katayama,¹ Miho Sekai,² Masakazu Hattori,³ Ichiro Miyoshi,⁴ Yoko Hamazaki,¹ and Nagahiro Minato^{1,2}

¹Department of Immunology and Cell Biology, Graduate School of Medicine, and ²Graduate School of Biostudies, Kyoto University, Kyoto; ³Graduate School of Science, Kitasato University, Kanagawa; and ⁴Center for Experimental Animal Science, Nagoya City University Graduate School of Medical Science, Nagoya, Japan

Rap family GTPases consist of multiple members with substantial functional redundancy. With the use of transgenic mice conditionally expressing a bona fide dominant-negative Rap1 mutant, *Rap1A17*, capable of inhibiting the activation of all Rap family members in B-lineage cells (mb.1-Rap1A17 Tg), we demonstrate that these mice show a defective generation of pre-B cells in bone marrow, resulting in a significant diminution of peripheral mainstream B cells. The effect

is attributed to the impaired survival and expansion of B-lineage progenitors in response to IL-7, despite normal IL-7R α expression. The pre-B cells from mb.1-Rap1A17 Tg mice showed a significantly reduced expression of *c-myc* and *E2A*, and the competence of IL-7 response was restored by the transduction of *c-myc*, but not by constitutively active (CA) *Stat5a*, CA *PI3K-p100*, or *bcl-2*. The residual follicular B cells with complete Cre-mediated recombination

proliferated normally in response to B-cell receptor stimulation and showed efficient germinal center reaction in vivo. These results show that endogenous Rap signaling plays a crucial role selectively in B-lineage cell development by sustaining the competence for IL-7 response, whereas it is dispensable for mature B-cell function. (Blood. 2009;114:1768-1775)

Introduction

B-cell development is a highly coordinated multistep process, including commitment and specification by a series of transcription factors such as *E2A*, *EBF1*, and *Pax5*, the rearrangement and expression of IgH and IgL genes, as well as proliferation and differentiation by stroma-derived cytokines such as IL-7.^{1,2} In the processes, various small GTPase signals play crucial and distinct roles.³ For instance, transgenic expression of a dominant-negative Ras (*RasN17*) results in the profound block of B-lineage progenitor (BLP) development before expression of pre-B-cell receptor (BCR), which is rescued by the expression of a dominant-active *c-Raf*.⁴ In agreement with the results, a recent report indicated that ERK-mediated transcriptional events are essential for the transition of pro-B to pre-B cells as well as pre-BCR-mediated pre-B-cell expansion,⁵ indicating an essential role of a classic Ras/Raf/ERK signaling pathway in the development of BLPs.⁶ Deficiency of both Rac1 and Rac2, however, causes the arrest of B-cell development at a later stage, ie, differentiation of immature B into transitional and mature B cells, suggesting that Rac signaling is crucial for BCR-mediated "positive selection."⁷

Rap GTPases, which belong to the Ras superfamily, consist of at least 5 highly related GTPases, Rap1 (a, b) and Rap2 (a, b, c).⁸ Both Rap1 and Rap2 GTPases share regulatory factors, Rap GTP/GDP exchange factors and Rap GTPase-activating proteins, including Spa-1, suggesting that they are under coordinate regulation.⁹ We previously reported that Rap signaling played a role in the control of proliferation of normal hematopoietic progenitors, in that deregulated Rap activation resulted in the development of a wide spectrum of leukemia with long latent periods.^{10,11} More recently, we reported that a T-lineage cell-specific expression of *Spa-1* transgene, which abrogated the endogenous activation of both Rap1 and Rap2 GTPases, caused a profound block of normal T-cell development at a pre-T-cell stage.¹² In stark contrast,

recent reports have shown that T-cell development is grossly normal in both Rap1a^{-/-} and Rap1b^{-/-} mice,^{13,14} strongly suggesting the substantial functional redundancy among the Rap family members in T-lineage cell progenitors. Similarly, it is reported that B-cell development is largely unaffected in Rap1a^{-/-} and Rap1b^{-/-} mice, although Rap1b^{-/-} mice show a selective diminution of marginal zone (MZ) B cells attributable to the perturbed trafficking, rather than the developmental defect.^{13,15} Considering the functional redundancy, however, the role of the Rap signaling in B-lineage cell development and function remains elusive.

In the current study, we have developed conditional transgenic (Tg) mice expressing a Rap mutant (*Rap1[S17A]*, or *Rap1A17* in short) in B-lineage cells (mb.1-Rap1A17). Unlike previously used Rap1N17 as a counterpart of RasN17, Rap1A17 exhibits a bona fide dominant-negative effect on the Rap family members in a physiologic setting.¹⁶ Here, we show that the development of pre-B cells in the bone marrow (BM) of mb.1-Rap1A17 Tg mice is profoundly impaired because of the defective IL-7 responsiveness, resulting in a significant diminution of peripheral mainstream B cells. We provide evidence that endogenous Rap signaling plays a crucial role in the development of BLPs by sustaining the expression of the competence genes for IL-7 response.

Methods

Mice

The *Rap1A17* transgene was produced by ligating 0.6-kilobase (kb) untagged *Rap1A17* cDNA into the *EcoRI* cloning site of the *pCALBIG* vector, which was created by ligating an *Ires-Egfp* fragment into the

Submitted March 27, 2009; accepted June 23, 2009. Prepublished online as *Blood* First Edition paper, June 30, 2009; DOI 10.1182/blood-2009-03-213371.

The publication costs of this article were defrayed in part by page charge

payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

© 2009 by The American Society of Hematology

EcoRI/ClaI sites of the *pCALBIG* vector (a gift from Dr D. Kitamura, Tokyo University of Science, Chiba, Japan). A purified *SfiI/SalI* fragment of *pCALBIG-Rap1A17* was injected into C57BL/6 (B6) × 129 F1 mouse zygote pronuclei to generate Rap1A17^f Tg mice. The Tg mice were backcrossed to B6 mice for more than 6 generations. The mb.1-Cre Tg mice¹⁷ were kindly provided by Dr M. Reth (Max Planck Institute of Immunobiology, Freiburg, Germany). B6 and Rag2^{-/-} mice were purchased from SLC and Central Institute of Experimental Animals, respectively. All mice were maintained under specific pathogen-free conditions at the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University. The animal experiments in this study were approved by the Review Board, the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University, Japan, and were performed according to the guidelines for animal experiments of Kyoto University.

Flow cytometric analysis and cell sorting

Multicolor flow cytometric analysis and cell sorting were done with the use of FACSCalibur (BD Biosciences) and FACSAriaII (BD Biosciences), as before.¹² Antibodies used were as follows: monoclonal antibodies to CD3ε, CD5, CD21, CD23, CD25, CD43, CD95, Mac-1, B220, c-kit, GL-7, IgM, IgD, and IgG1 were purchased from BD Biosciences, and Alexa Fluor 488-conjugated PNA was purchased from Invitrogen.

Cells and culture

The WEHI231 and 2E8 B-cell lines were maintained with RPMI 1640 supplemented with 10% fetal calf serum, 50 mM 2-mercaptoethanol, 100 mM nonessential amino acids, 1 mM sodium pyruvate, and antibiotics, in the presence of IL-7 (10 ng/mL) for the latter. Primary mouse embryonic fibroblasts (MEFs) and the PLAT-E cell line, a kind gift from Dr T. Kitamura (University of Tokyo, Tokyo, Japan), were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum and antibiotics. B220⁺ cells in BM were enriched by depleting other cell types with a cocktail of antibodies (anti-Thy1, anti-Gr-1, anti-Mac1, and anti-Ter117) and anti-rat IgG-conjugated magnetic beads (Invitrogen) and were cultured (5 × 10⁵/mL) in the presence of IL-7 (10 ng/mL) for various days. Splenic B220⁺ CD23⁺ follicular (FO) B cells were purified by a cell sorter and cultured in the presence of anti-IgM (20 μg/mL), anti-IgM plus anti-CD40 (5 μg/mL), anti-CD40 plus IL-4 (10 ng/mL), or LPS (10 μg/mL) plus IL-4 for 3 days. Cell proliferation was determined by the incorporation of ³H-TdR for 12 hours. For the cell adhesion assay, cells were plated on the microwells coated with fibronectin (1 μg/mL), and, after a gentle wash 3 times, the adherent cells were released with ethylenediaminetetraacetic acid (5 mM) and enumerated with a FACSCalibur.

Immunoblotting and pull-down assay

Cells were lysed with ice-cold lysis buffer (150 mM NaCl, 50 mM Tris-HCl [pH 7.6], 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na₃VO₄, 10 mM NaF, 2 mg/mL leupeptin, 2 mg/mL aprotinin). Immunoblotting was done with anti-c-myc (Cell Signaling Technology) and anti-GAPDH (BD Biosciences). RapGTP was detected by pull-down assay with the use of a GST fusion protein of the Rap binding domain of RalGDS, followed by immunoblotting with anti-Rap1 or anti-Rap2 antibody (Santa Cruz Biotechnology), as described previously.¹²

Polymerase chain reaction

Cre-mediated recombination of the *Rap1A17^f* transgene was detected by genomic polymerase chain reaction (PCR) with the following primers: *CAG*-sense, atgattaattcagcgaacg; *Rap1A17*-antisense, atcattaacgtagactgagc. For quantitative reverse transcription (qRT)-PCR, total RNA was extracted from the purified B220⁺ CD25⁺ pre-B-cell populations in the BM with a NucleoSpin RNA kit (Macherey-Nagel) followed by cDNA synthesis with SuperScript III (Invitrogen). Real-time PCR was performed with a LightCycler SYBR Green I marker kit on a LightCycler instrument (Roche). The primer pairs were as follows: *E2A* (*E12*): sense, cagacgaggacgaggacgac, antisense, ctgctttgttccagttctg; *E2A* (*E47*): sense, cagatgaggtgctgtccctgga, antisense, ctgctttgttccagttctg; *Rag1*: sense, aggcctgtggagcaaggta, antisense, gctcagggtagacggcaag;

Rag2: sense, tgccaaaataagaagagtatttcac, antisense, gggacatttttgattgtgaatagg; *Ebf1*: sense, ccaactcacctatgccatt, antisense, ggggaggctttagatgagg; *Pax5*: sense, ttgttgacgtcagctctgt, antisense, ggggaacctccaagaatcat; *CD19*: sense, gactcttctccaatgctgag, antisense, atgggggctcagggaagtc; *Oct1a*: sense, gctgggaagtcttaggagtc, antisense, ttgttgacgtcagctctgt; *c-myc*: sense, cgaactctggtgcataaactg, antisense, gaaccttctcttagctctca; *Cyclophilin*: sense, gacgaaggtagccagtcacaag, antisense, aatcaggcctgtggaatgtgag. The transcripts of each gene were normalized to those of cyclophilin.

Retrovirus production and infection

cDNA of constitutively active (CA) *Stat5a* (provided by Dr K. Ikuta, Virus Research Institute, Kyoto University, Kyoto, Kapan), *CA PI3K-p100* (provided by Dr S. Koyasu, Keio University School of Medicine, Tokyo, Japan), *Bcl-2* and *c-Myc* (provided by Dr H. Ariga, Hokkaido University, Sapporo, Japan) were subcloned into a *pMCs Ires-Egfp* (MIG) retroviral vector. PLAT-E packaging cells were transfected with the retrovirus plasmids, and the culture supernatants were harvested for the recombinant retrovirus. Cells were infected with the supernatants at 800g centrifugation in the presence of 4 mg/mL polybrene at 32°C for 60 minutes. Infected cells were identified by GFP with the use of FACSCalibur.

Immunization and immunostaining

Mice were immunized with 5 × 10⁸ sheep red blood cells (SRBCs) intraperitoneally. Immunostaining was performed as described before.¹⁸ Spleens were snap-frozen in optimum cutting temperature compound, and frozen sections were fixed with acetone. After blocking with BSA (1% in PBS), samples were stained with the indicated antibodies followed by secondary antibodies, mounted in Mowiol (Calbiochem). Images were acquired with the use of fluorescence microscopy (Axiovert 200M) equipped with AxioCam MRm, Fluor 2.5×/0.12 or 5×/0.25 numerical aperture objective lens (Carl Zeiss) and analyzed with AxioVision Version 4.6 (Carl Zeiss). Digital images were processed using Adobe Photoshop CS2 (Adobe Systems).

Statistical analysis

Statistical analysis was performed with the use of the 2-tailed Student *t* test.

Results

Defective development of pre-B cells in mb.1-Rap1A17 Tg mice

To confirm the dominant-negative effect of Rap1A17 in B-lineage cells, the WEHI231 B-cell line was infected with empty retrovirus (MIG) or MIG containing *Rap1A17*, and the sorted GFP⁺ cells were stimulated with anti-IgM antibody. Although control WEHI231 cells showed potent activation of Rap1 and Rap2, those infected with MIG/Rap1A17 showed a markedly compromised activation of both (Figure 1A). We then generated Rap1A17^f Tg mice, which harbor a cassette containing *Rap1A17* downstream of a loxP-flanked intervening sequence (*blasticidin S deaminase*) preventing the transcription of *Rap1A17* by CAG (chicken actin/globin hybrid) promoter (Figure 1B). Because preliminary experiments showed that the addition of tags to *Rap1A17* resulted in the compromised dominant-negative effect, untagged cDNA was used. Infection of the MEF derived from Rap1A17^f Tg mice with Cre-containing adenovirus in vitro resulted in the deletion of *blasticidin S deaminase* and the transcription of *Rap1A17* as expected, causing a substantial reduction of endogenous RapGTP (Figure 1B). Rap1A17^f Tg mice were then crossed with mb.1-Cre Tg mice to generate double Tg (mb.1-Rap1A17) mice. In the BM of adult mb.1-Rap1A17 Tg mice, the proportions as well as the absolute numbers of B-lineage cells were markedly and selectively diminished compared with Rap1A17^f littermates (Figure 1C-D). Among the B-lineage cells of distinct developmental stages, pre-B (CD43^{low}

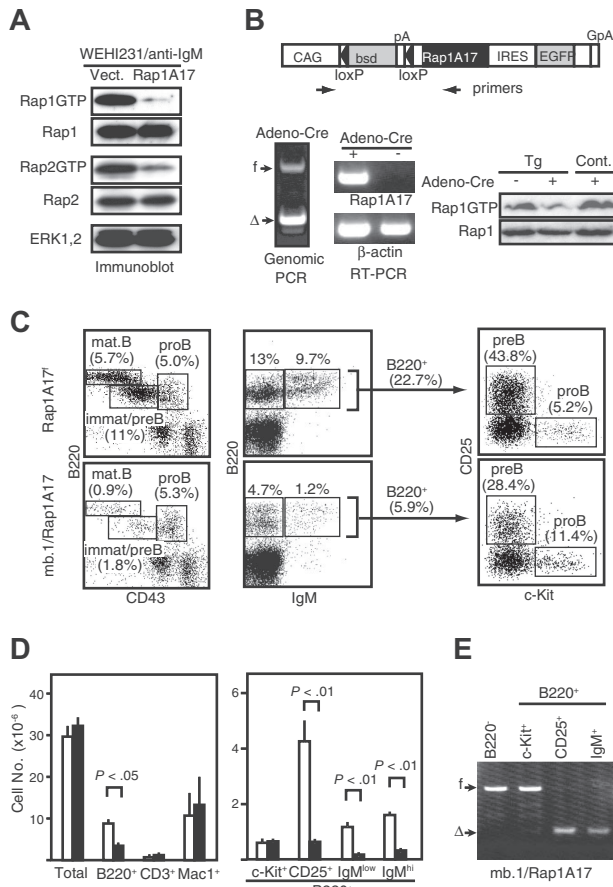


Figure 1. Defective development of pre-B cells in the adult BM of mb.1-Rap1A17 Tg mice. (A) The WEHI231 B-cell line was infected with empty MIG or MIG containing untagged *Rap1A17* cDNA. Sorted GFP⁺ cells were stimulated with anti-IgM antibody (20 μ g/mL) for 10 minutes, and the lysates were immunoblotted with the indicated antibodies. Rap1GTP and Rap2GTP were assessed by a pull-down assay with GST-RalGDS. (B) A schematic representation of a transgenic *Rap1A17* plasmid (top). MEFs from the Tg mice were infected with empty or *Cre*-containing adenovirus, *Cre*-mediated recombination, and *Rap1A17* transcripts were assessed by genomic and RT-PCR, respectively, using an indicated primer set. f indicates floxed; Δ , deleted. Rap1GTP was also assessed (bottom). (C) BM cells from mb.1-Rap1A17 Tg mice and Rap1A17^f littermates were multicolor analyzed with the indicated antibodies with the use of FACS Calibur. Cell proportions of the indicated gates are shown. (D) Absolute numbers of each cell fraction in the BM from Rap1A17^f (□) and mb.1-Rap1A17 (■) Tg littermates. Means and SEs of 5 mice are indicated. (E) Cell populations of the indicated phenotypes were sorted from the BM of mb.1-Rap1A17 Tg mice, and *Cre*-mediated recombination was assessed by genomic PCR.

CD25⁺, immature, and mature B (CD43⁻ IgM⁺) cells were all diminished significantly, whereas pro-B cells (CD43^{high} c-Kit⁺) were unaffected (Figure 1C-D). These B-lineage cells hardly showed detectable GFP expression, possibly in part because of the deletion of the cells strongly expressing the transgene. We therefore directly examined the *Cre*-mediated gene recombination with genomic PCR analysis. It was confirmed that the pre-B and immature/mature B cells showed nearly complete *Cre*-mediated recombination of the transgene, whereas pro-B cells showed no evidence of the recombination (Figure 1E). We thus conclude that endogenous Rap signaling is crucial for the development of pre-B cells in adult BM, although its possible role in pro-B-cell development remains to be seen.

Impaired IL-7 response of BLPs expressing *Rap1A17*

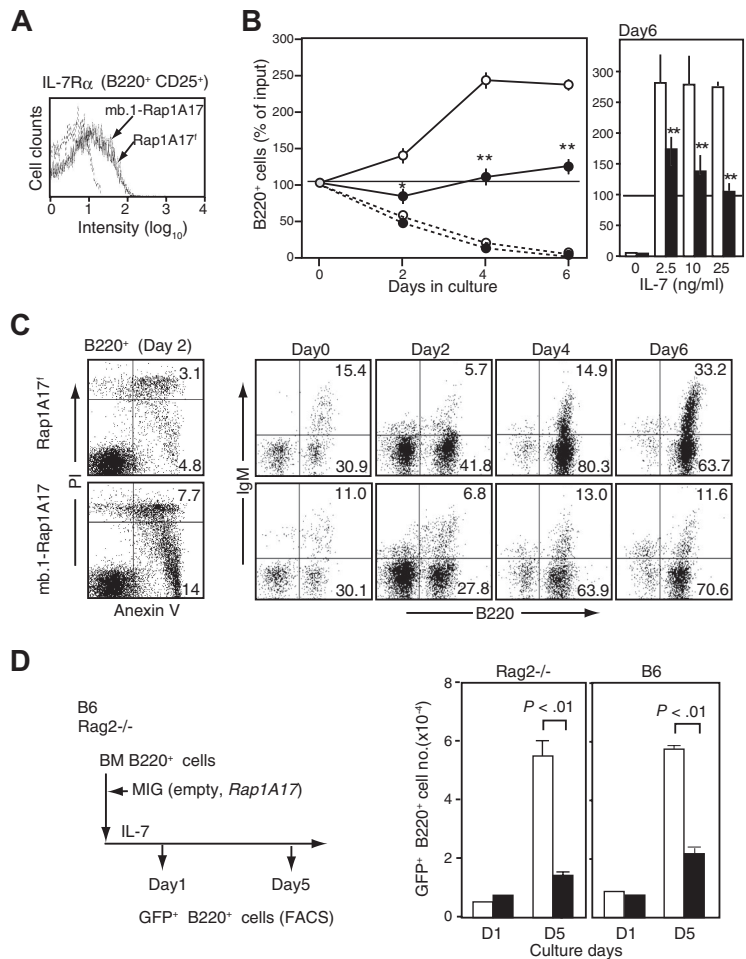
We then examined the responsiveness of BLPs to IL-7, an essential factor for early B-cell development in mice. Pre-B cells from mb.1-Rap1A17 Tg mice showed comparable expression of IL-7R α

with those from Rap1A17^f littermates (Figure 2A). Nonetheless, BM B220⁺ cells from mb.1-Rap1A17 Tg mice were hardly increased in the presence of optimal doses of IL-7 (2.5–10 ng/mL) in vitro until 6 days, whereas control BM B220⁺ cells showed a 2.5-fold expansion (Figure 2B). Because the starting populations included pro-B cells without *Cre*-recombination, the effect might even be underrated. The response showed no tendency for the restoration by increasing the doses of IL-7 up to 25 ng/mL (Figure 2B). Furthermore, the B220⁺ cells from mb.1-Rap1A17 Tg mice showed a significantly increased apoptosis on as early as day 2 in the presence of IL-7 compared with the control cells, and the eventual generation of IgM⁺ cells was also compromised (Figure 2C). Pre-BCR signaling plays a significant cooperative role in the IL-7-mediated proliferation of pre-B cells, in particular at the suboptimal doses of IL-7.¹⁹ To examine the possible effect of pre-BCR on the defective IL-7 response, we retrovirally transduced *Rap1A17* to the BM B220⁺ cells from normal B6 and Rag2^{-/-} mice and cultured them in the presence of IL-7. The Rag2^{-/-} B220⁺ cells expressing *Rap1A17* showed a markedly impaired expansion to the extent comparable with *Rap1A17*^f B6 B220⁺ cells (Figure 2D). These results suggest that the BLPs in mb.1-Rap1A17 Tg mice are incompetent for survival and proliferation in response to IL-7 irrespective of pre-BCR.

Competence for IL-7 response of the pre-B cells from mb.1-Rap1A17 Tg mice is restored by *c-myc* transduction

We first examined the possibility that Rap1A17 might interfere with IL-7R signaling per se. JAK3/Stat5 and PI3K/AKT pathways as well as some Bcl-2 family members are shown to be involved in the IL-7R signaling of BLPs.²⁰ Because the pre-B cells in mb.1-Rap1A17 Tg mice were not feasible for direct biochemical analysis because of the small cell numbers, we addressed the possibility by retroviral transduction of CA *Stat5a*, CA *PI3K-p100*, or *Bcl-2*. Neither of them, however, affected the IL-7 response of BM B220⁺ cells from mb.1-Rap1A17 Tg mice (Figure 3A). The transduction of CA *Stat5a* in control pre-B cells resulted in the significant diminution of IL-7 response (Figure 3A), suggesting that overactivation of *Stat5a* might be rather inhibitory. Thus, a partial contribution of this effect to the lack of restoration by *Stat5a* could not be ruled out. Nonetheless, the results raised an alternative possibility that Rap1A17⁺ pre-B cells might be intrinsically incompetent for IL-7 response. Crucial competence factors for IL-7 response include Myc and E2A proteins, the overall level of which are major forces driving the survival and expansion of BLPs.^{21–23} We then isolated the pre-B cells (B220⁺CD25⁺) from mb.1-Rap1A17 Tg and control littermate mice and directly examined the expression of *c-Myc* and *E2a* (*E12*, *E47*) without any culture with qRT-PCR. The pre-B cells from mb.1-Rap1A17 Tg mice showed a significant decrease in *c-myc* and more prominently in *E2a* compared with the control pre-B cells (Figure 3B); *N-myc* expression was below the detection limit in both groups (data not shown). In agreement with the findings, the target genes of *E2a*, *Rag1*, and *Rag2*, were also diminished significantly, although other downstream B-lineage-specific genes were reduced only insignificantly (Figure 3B). Furthermore, retroviral expression of *c-Myc* in the pre-B cells from mb.1-Rap1A17 Tg mice significantly restored the IL-7-mediated expansion, whereas it was without effect on the response of control pre-B cells (Figure 3C). It was confirmed that the retroviral transduction resulted in a 9-fold increase of *c-myc* transcripts as judged by qRT-PCR. To confirm the results, we retrovirally transduced *Rap1A17* in an IL-7-dependent B-cell line (2E8). The sorted GFP⁺ cells were cultured in the absence of IL-7

Figure 2. Impaired IL-7 response of the BM BLPs from mb.1-Rap1A17 Tg mice. (A) BM cells from Rap1A17^f (solid line) and mb.1-Rap1A17 (fine dotted line) Tg mice were 3-color stained with anti-B220, anti-CD25, and anti-IL-7 α antibodies and analyzed with FACSCalibur. IL-7R α -staining profiles in a B220⁺ CD25⁺ gate are indicated. The coarse dotted line indicates the control staining. (B) The B220⁺ BM cells from Rap1A17^f (○) and mb.1-Rap1A17 Tg (●) mice were cultured in the presence (solid lines) or absence (dotted lines) of IL-7 (10 ng/mL), and B220⁺ cell numbers were determined at the indicated days (left). In independent experiments, the cells were cultured in the presence of various doses of IL-7 (0 ~ 25 ng/mL) for 6 days (right). The mean percentages and SEs of the viable B220⁺ cell recoveries in triplicate cultures are indicated. **P* < .05; ***P* < .01. (C) The aliquot cells of the cultures (IL-7 at 10 ng/mL) were multicolor analyzed with the indicated antibodies. The percentages of cells in the quadrants are indicated. (D) B220⁺ cells sorted from the BM of normal B6 and Rag2^{-/-} mice were infected with empty (□) or Rap1A17-containing (■) MIG and cultured in the presence of IL-7 (10 ng/mL). On days 1 and 5, the numbers of GFP⁺ B220⁺ cells were determined. The means and SEs of triplicate cultures are indicated.



for 24 hours followed by the stimulation with IL-7 (Figure 3D). The Rap1A17⁺ cells showed a diminished expression of c-Myc protein and failed to survive and expand thereafter in the presence of IL-7, whereas the control GFP⁺ cells initiated the proliferation (Figure 3B). The results suggest that the impaired survival and proliferation of Rap1A17⁺ pre-B cells are attributable to the intrinsic defect in the competence for IL-7 response in part via c-Myc.

Diminished mainstream B cells in the peripheral tissues

In agreement with the impaired pre-B-cell development in BM, the numbers of newly emerged T1 (B220⁺ CD21^{low} CD23⁻) and FO (IgM⁺ CD21^{low} CD23⁺) B cells in the spleen as well as B2 cells (B220⁺ Mac1⁻ CD5⁻) in the peritoneal cavity were markedly diminished in mb.1-Rap1A17 Tg mice (Figure 4A-B). It was unlikely that the effects were due to the perturbed lymphoid tissue homing because B cells in the circulation were also diminished (Figure 4A). The numbers of MZ B (IgM^{high} CD21^{high} CD23⁻) cells were unchanged; however, most of them showed no Cre-mediated recombination; thus, the effect evaded the evaluation (Figure 4C). Peritoneal B1 (B220⁺ Mac1⁺/CD5⁺) cells in mb.1-Rap1A17 Tg mice, most of which showed Cre-mediated recombination, exhibited significantly reduced expression of B220 compared with the control B1 cells, albeit the number was unchanged (Figure 4A-B). Immunostaining analysis confirmed that the spleens of mb.1-Rap1A17 Tg mice showed significantly reduced numbers of follicles with enlarged red pulps (Figure 4D); because the spleen sizes were smaller, the estimation even was an underestimate.

However, the mean follicular size in mb.1-Rap1A17 Tg mice remained comparable with that in control littermates (Figure 4D).

Rap signaling is dispensable for the function of FO B cells

Given that the residual FO B cells in mb.1-Rap1A17 Tg mice showed nearly complete Cre-mediated recombination, we finally examined the role of Rap signaling in their functioning. B220⁺ CD23^{high} cells sorted from mb.1-Rap1A17 Tg mice showed significantly compromised β 1-integrin-mediated cell adhesion in agreement with the defective Rap activation; however, the proliferation in response to BCR stimulation was not impaired but rather tended to be enhanced compared with those from control littermates, particularly in the absence of anti-CD40 antibody (Figure 5A). These cells also developed comparable or even more IgG1-bearing B cells after IL-4 plus anti-CD40 or LPS stimulation, although IgG1⁺ cells were negligible before the stimulation (Figure 5B). Furthermore, on immunization with SRBCs, most of the follicles in the spleens of mb.1-Rap1A17 Tg mice showed typical germinal center (GC) reaction, albeit the absolute numbers of follicles were diminished. Actually, the relative proportion of GC B cells to total B cells was rather increased in mb.1-Rap1A17 Tg mice, and the total GC B-cell numbers reached nearly comparable levels to those in control littermates after immunization (Figure 5C). These results indicate that endogenous Rap signaling is dispensable, or may be rather inhibitory, for the function of mature FO B cells in response to antigens.

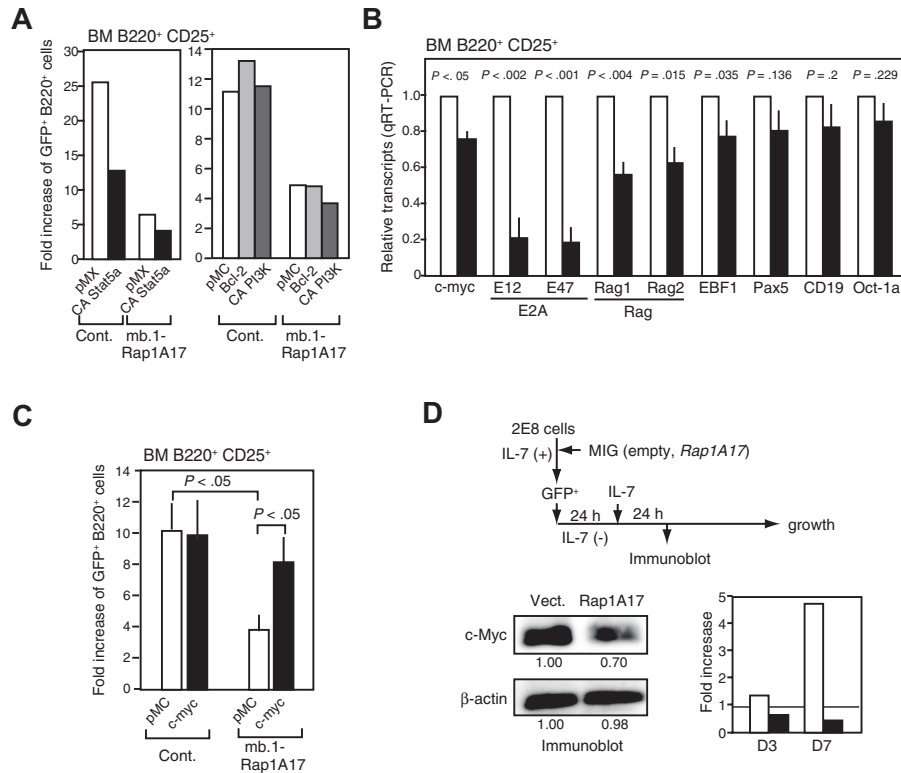


Figure 3. Reduced expression of *c-myc* and *E2A* in the pre-B cells from mb.1-Rap1A17 Tg mice and restoration of IL-7 response by *c-myc*. (A) BM B220⁺ cells from Rap1A17^f (□) and mb.1-Rap1A17 (■) Tg littermates were infected with empty retrovirus (pMX or pMC), pMX-CA *Stat5a*, pMC-*bcl-2*, or pMC-CA *PI3K* and cultured in the presence of IL-7. The cells were harvested on days 1 and 5, and the numbers of GFP⁺ B220⁺ cells were assessed by flow cytometric analysis. Fold increases (D5/D1) in the GFP⁺ B220⁺ cell numbers are indicated. Similar results were obtained in 2 independent experiments, and the means are indicated. (B) B220⁺ CD25⁺ cells were isolated from the BM of Rap1A17^f (□) and mb.1-Rap1A17 (■) Tg littermates, RNAs were extracted immediately, and quantitative RT-PCR was performed for the indicated genes. The means and SEs of the relative expression in the cells from mb.1-Rap1A17 to those from Rap1A17^f littermates (3 mice each) are indicated. (C) B220⁺ cells enriched from the BM of Rap1A17^f (□) and mb.1-Rap1A17 (■) Tg littermates were infected with empty MIG or MIG containing *c-myc*, and cultured in the presence of IL-7. The means and SEs of fold increases in the GFP⁺ B220⁺ cell numbers in 3 independent experiments are indicated. (D) The 2E8 cells were infected with empty MIG (□) or MIG containing *Rap1A17* (■), and the sorted GFP⁺ cells were starved for IL-7 for 24 hours followed by the restimulation with IL-7. On day 1, the cells were lysed and immunoblotted with the indicated antibodies (left). Relative signal intensities are shown. The fold increases of the cells to the original input were also determined on days 3 and 7 (right). The means of 2 independent experiments with similar results are shown.

Discussion

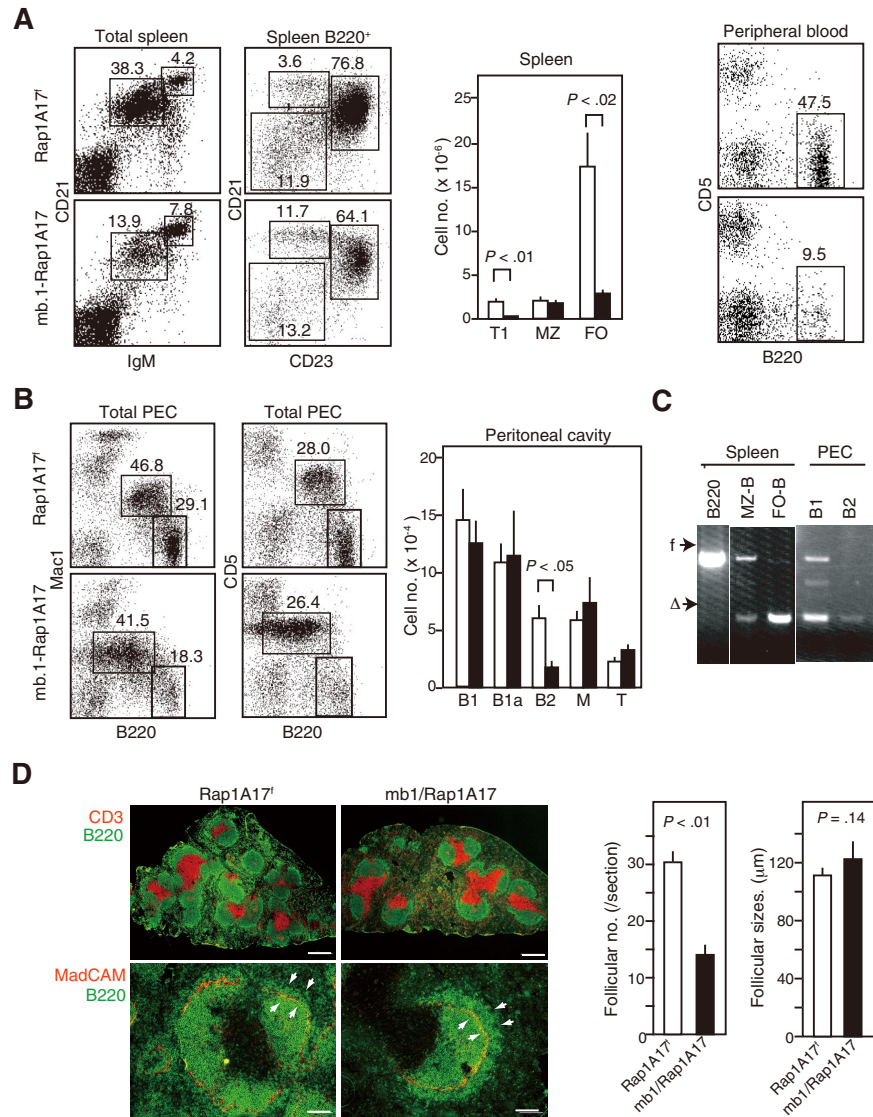
Despite the high structural similarity to Ras, Rap GTPases may show signaling modes different from classic Ras and may play distinct roles in various cell types.^{8,11,24} A complication of the functional studies on Rap signaling in vivo has been the presence of multiple, highly homologous members encoded by distinct genes with substantial functional redundancy. Although the Rap1N17 mutant has been used widely to inhibit Rap activation, it does not function as a true dominant-negative form and may not necessarily affect the endogenous Rap activation via physiologic stimuli.¹⁶ Recently, it has been indicated that Rap1A17 mutant shows a bona fide dominant-negative effect by forming a stable nucleotide-free complex with the major GTP/GDP exchange factors and effectively inhibits the activation of Rap family members in a physiologic setting.¹⁶ In current study, we investigated the role of Rap signaling in the development and function of B cells in vivo with the use of mb.1-Rap1A17 Tg mice conditionally expressing *Rap1A17* in B-lineage cells.

The mb.1-Rap1A17 Tg mice showed a marked diminution of BM pro-B cells, whereas pro-B-cell development was unaffected. Although the pre-B cells revealed complete Cre-mediated recombination, it was hardly detected in pro-B cells; thus the possible effects on pro-B cells remained to be seen. It has been reported that the recombination effect of mb.1-Cre becomes detectable from the pro-B cell stage in the mice expressing certain loxP-flxed transgenes¹⁷; therefore it seems that the

current *Rap1A17* transgene requires relatively higher Cre activity for the recombination. In either case, the results disclose that endogenous Rap signaling plays a crucial role in pre-B-cell development. The pre-B cells from mb.1-Rap1A17 Tg mice were defective in the IL-7 response in vitro, despite normal IL-7R α expression. Because the expression of *Rap1A17* in the primary Rag2^{-/-} BM B220⁺ cells also resulted in the inhibition of IL-7-mediated expansion comparable with those in B6 BM B220⁺ cells, it was unlikely that the effect could be attributable to the perturbed pre-BCR signaling cooperating with IL-7. The survival and proliferation of pre-B cells by IL-7 are mediated by the signaling of JAK3/Stat5, PI3K/AKT, as well as Bcl-2 pathways²⁰; however, the expression of CA *Stat5a*, CA *PI3K-p100*, or *Bcl-2* failed to restore the IL-7 response of the pre-B cells from mb.1-Rap1A17 Tg mice. Furthermore, we previously reported that the development of $\gamma\delta$ T cells, which also depend on IL-7,²⁵ was unaffected at all in lck-Spa-1 Tg mice¹²; we have confirmed the same results in lck-Rap1A17 Tg mice (K. Wakae and N.M., unpublished observation, May 2009). The retroviral expression of CA *Stat5a* is reported to restore the defective $\gamma\delta$ T-cell development in IL-7^{-/-} mice.²⁶ These results collectively suggest that the impaired survival and proliferation of Rap1A17⁺ pre-B cells in the presence of IL-7 may be attributable to the intrinsic incompetence rather than to the interference with IL-7R signaling.

The survival and proliferation of BLPs are suggested to depend crucially on the overall levels of Myc proteins.²¹ Current results have shown that the pre-B cells from mb.1-Rap1A17 Tg mice showed a reduced expression of *c-Myc*, suggesting that the

Figure 4. Diminished follicular B cells in mb.1-Rap1A17 Tg mice. (A) Spleen cells from Rap1A17⁺ and mb.1-Rap1A17 Tg littermates were multicolor analyzed with the indicated antibodies with the use of FACSCalibur (left). The mean numbers and SEs of T1 (B220⁺ CD21^{low} CD23⁻), MZ (B220⁺ CD21^{high} CD23⁻), and FO (B220⁺ CD21^{low} CD23⁺) B cells in the spleens of Rap1A17⁺ (□) and mb.1-Rap1A17 (■) Tg littermates (5 mice each) are indicated (middle). Peripheral blood cells were also analyzed with the indicated antibodies (right). (B) Peritoneal exudate cells from Rap1A17⁺ (□) and mb.1-Rap1A17 (■) Tg mice were 2-color analyzed with the indicated antibodies (left), and the numbers of B1 (B220⁺ Mac1⁻), B1a (B220⁺ CD5⁺), and B2 (B220⁺ Mac1⁻ CD5⁻) cells, as well as macrophages and T cells, are indicated (right). The means and SEs of 5 mice in each group are shown. (C) Indicated B-cell subsets sorted from the spleen and peritoneal cells from mb.1-Rap1A17 Tg mice were assessed for Cre-mediated recombination by genomic PCR. (D) The spleens from mb.1-Rap1A17 (■) and Rap1A17⁺ (□) littermates were immunostained with the indicated antibodies (Cy3-anti-CD3 and Alexa Fluor 488 anti-B220). ■ indicates MZ, and bars indicate 400 μm (top) and 100 μm (bottom). The mean follicular numbers in 4 sections of central regions and the mean major axis (in μm) of 60 follicles were measured.



endogenous Rap signaling plays a role in sustaining the expression of *c-Myc*. Importantly, the defective IL-7 response was restored significantly by the transduction of *c-Myc*, indicating that the reduced *c-myc* expression accounted for the incompetence for IL-7 response at least in part. In agreement with the results, the retroviral expression of *Rap1A17* in an IL-7-dependent B-cell line (2E8) resulted in the decreased expression of *c-Myc* protein and the concomitant impairment of IL-7-mediated survival and proliferation. In addition to *c-Myc*, pre-B cells from mb.1-Rap1A17 Tg mice showed even more pronounced diminution of *E2a* expression. The basic helix-loop-helix transcription factor E2A is an essential factor for B-lineage cell commitment and specification.^{27,28} Recent studies, however, have shown that E2A activity continues to be required for the development of pre-B cells as well.^{29,30} Interestingly, although the introduction of early B-cell factor (*Ebf*) gene can restore the generation of pre-B cells from E2A^{-/-} fetal liver, the E2A^{-/-} EBF⁺ fetal liver pre-B cells remain to be poorly responsive to IL-7, despite the normal expression of IL-7Rα.²² The results indicate that E2A functions as a crucial competence factor of IL-7 response in pre-B cells, for which higher E2A activity may be needed than for B-lineage cell commitment. In agreement with the findings, E2A^{+/-} mice show a 2-fold reduction of pre-B cells, whereas the numbers of pro-B cells are unaffected.³⁰ It is shown

that the effect of E2A on IL-7 responsiveness is mediated in part by supporting *myc* expression.²² Therefore, it may be plausible that the reduced expression of *E2a* may underlie the defective development of pre-B cells in mb.1-Rap1A17 Tg mice. A direct attempt to restore the *E2a* expression in the pre-B cells, however, was unsuccessful because of the well-recognized lethal effect of *E2a* overexpression.^{22,23} In addition, the 2E8 B-cell line showed little E2A expression in the current experimental conditions, albeit the Rap1A17 expression resulted in *c-Myc* depression. Thus, it remains to be verified whether E2A is involved directly in the control of *c-myc* expression levels by endogenous Rap signaling in the primary pre-B cells.

The defective development of pre-B cells resulted in the markedly diminished generation of mainstream B cells in mb.1-Rap1A17 Tg mice as expected. It was reported that both Rap1a^{-/-} and Rap1b^{-/-} mice showed normal numbers of mainstream B cells^{14,15}; thus, it is strongly suggested that functionally redundant, multiple Rap family members are involved in the B-cell development as well. Curiously, although the residual FO B cells showed the complete Cre-mediated recombination, MZ B cells showed only inefficient recombination, and their development was unaffected. The results may suggest that the mb.1-promoter activation in the progenitors of MZ B cells is weaker than that in

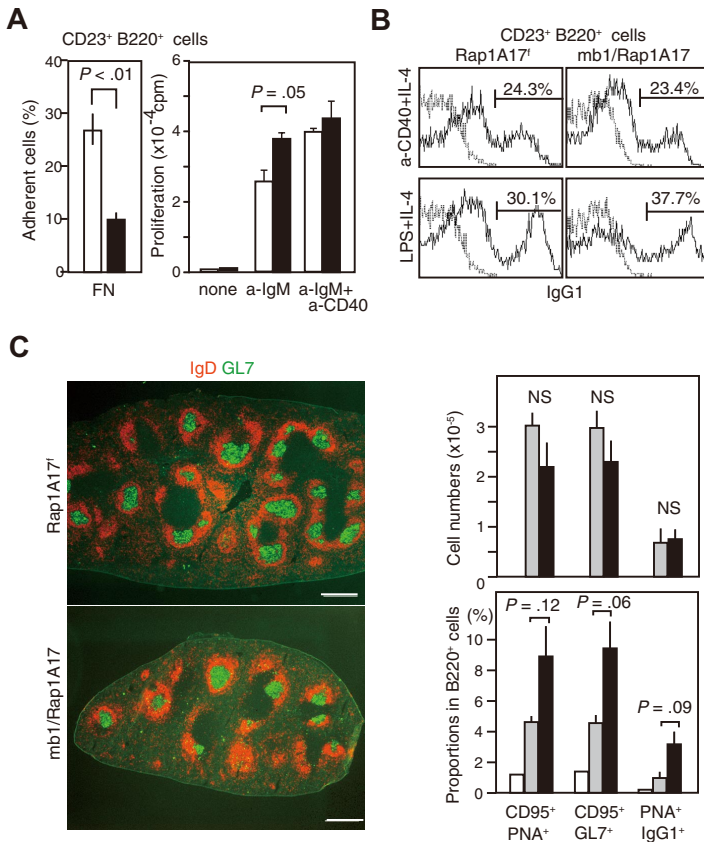


Figure 5. FO B cells in mb.1-Rap1A17 Tg mice show normal function with even enhanced efficiency of GC B-cell generation. (A) FO ($B220^+ CD23^{\text{high}}$) B cells sorted from Rap1A17^{-/-} (□) and mb.1-Rap1A17 Tg littermates were plated on the fibronectin (FN)-coated wells for 30 minutes, and the adherent cell proportion was assessed (left). The aliquots of the cells were cultured in the presence of anti-IgM or anti-IgM plus anti-CD40 (5 $\mu\text{g}/\text{mL}$ each) for 3 days followed by the pulse with ³H-TdR. The means and SEs of triplicate cultures are indicated. (B) FO B cells sorted from Rap1A17^{-/-} and mb.1-Rap1A17 Tg littermates before (fine lines) and after (solid lines) the culture in the presence of IL-4 (10 ng/mL) plus anti-CD40 or LPS (10 $\mu\text{g}/\text{mL}$ each) for 4 days were analyzed for anti-IgG1 expression with FACSCalibur. The proportions of IgG1⁺ cells are indicated. (C) Rap1A17^{-/-} and mb.1-Rap1A17 Tg littermates were injected intraperitoneally with SRBCs (5×10^8). Six days later, the spleens were immunostained with the indicated antibodies (left). The pictures at the widest transverse sections are indicated (PE-anti-IgD and Alexa Fluor 488-anti-GL7). Bars indicate 400 μm . The spleen cells were 3-color analyzed with anti-B220, anti-CD9, and PNA, and the proportions in total B220⁺ cells as well as the cell numbers of the indicated phenotypes are shown. □ indicate unimmunized normal mice; ■, immunized Rap1A17^{-/-} mice; ■, immunized mb.1-Rap1A17 mice. The means and SEs of 5 mice are indicated (right).

the progenitors of FO B cells. Although the peritoneal B1 cell number was unaffected either, they showed apparently lower expression of B220 than the control B1 cells. It may be possible that most B1 cells in mb.1-Rap1A17 Tg mice represent those of fetal origin, whose generation is independent of IL-7.³¹ The residual FO B cells showed a normal or even enhanced BCR-mediated proliferation response as well as IgG1 class switching in vitro. Reflecting the diminished FO B cells, the numbers of splenic follicles were significantly reduced; interestingly, however, the follicular sizes remained comparable with those in control spleens. In addition, on antigen immunization in vivo, mb.1-Rap1A17 Tg mice showed a highly efficient GC reaction, generating almost comparable numbers of GC B cells with control littermates. Thus, under the circumstance of diminished FO B cells, sustaining the follicular sizes may be more crucial for the effective immune response in vivo than keeping their numbers. In either case, albeit Rap GTPases are activated in mature B cells via BCR stimulation,^{32,33} Rap signaling is dispensable for the function of FO B cells in vivo; a possible negative effect of Rap signaling on GC reaction remains to be verified.

We have previously reported that endogenous Rap signaling is crucial in the thymic $\alpha\beta$ T-cell development; thus, abrogation of Rap signaling results in a profound block of pre-T-cell development because of the failure of p53-mediated death-rescue via pre-TCR.¹² Both Myc and E2A play important roles also in the early T-cell development^{34,35}; it remains to be investigated whether the role of Rap signaling in T-lineage cells also involves the control of these factors. Nonetheless, these results collectively indicate that Rap family GTPases play an essential role in the development of both pre-T and pre-B cells. Intriguingly, constitutive activation of

Rap signaling results in the leukemic transformation of immature T cells.³⁶ Possible effects of deregulated Rap activation on the leukemia genesis of B-lineage cells are currently under investigation.

Acknowledgments

We thank Dr M. Reth for providing the mb.1-Cre transgenic mice, Dr K. Ikuta for the CA Stat5a plasmid, Dr H. Ariga for the c-myc cDNA, Dr S. Koyasu for the CA PI3K-p100 cDNA, and Dr T. Kitamura for the PLAT-E cells and pMCs-IG vector. We also thank Dr Y. Agata for the helpful discussion.

This work was supported by grants from the Ministry of Education, Culture, Science, Sports, and Technology of Japan (N.M. and Y.H.).

Authorship

Contribution: Y.K. performed animal experiments and immunologic analysis; M.S. performed cell preparation and qPCR analysis; M.H. designed the research; I.M. produced the transgenic mice; Y.H. performed immunostaining analysis; and N.M. designed research and wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Nagahiro Minato, Graduate School of Medicine, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan; e-mail: minato@imm.med.kyoto-u.ac.jp.

References

- Nutt SL, Kee BL. The transcriptional regulation of B cell lineage commitment. *Immunity*. 2007;26(6):715-725.
- Northrup DL, Allman D. Transcriptional regulation of early B cell development. *Immunol Res*. 2008;42(1-3):106-117.
- Scheele JS, Marks RE, Boss GR. Signaling by small GTPases in the immune system. *Immunol Rev*. 2007;218:92-101.
- Iritani BM, Forbush KA, Farrar MA, Perlmutter RM. Control of B cell development by Ras-mediated activation of Raf. *EMBO J*. 1997;16(23):7019-7031.
- Yasuda T, Sanjo H, Pages G, et al. Erk kinases link pre-B cell receptor signaling to transcriptional events required for early B cell expansion. *Immunity*. 2008;28(4):499-508.
- Nagaoka H, Takahashi Y, Hayashi R, et al. Ras mediates effector pathways responsible for pre-B cell survival, which is essential for the developmental progression to the late pre-B cell stage. *J Exp Med*. 2000;192(2):171-182.
- Walmsley MJ, Ooi SK, Reynolds LF, et al. Critical roles for Rac1 and Rac2 GTPases in B cell development and signaling. *Science*. 2003;302(5644):459-462.
- Raaijmakers JH, Bos JL. Specificity in Ras and Rap signaling. *J Biol Chem*. 2009;284(17):10995-10999.
- Hattori M, Minato N. Rap1 GTPase: functions, regulation, and malignancy. *J Biochem*. 2003;134(4):479-484.
- Ishida D, Kometani K, Yang H, et al. Myeloproliferative stem cell disorders by deregulated Rap1 activation in SPA-1-deficient mice. *Cancer Cell*. 2003;4(1):55-65.
- Minato N, Kometani K, Hattori M. Regulation of immune responses and hematopoiesis by the Rap1 signal. *Adv Immunol*. 2007;93:229-264.
- Kometani K, Moriyama M, Nakashima Y, et al. Essential role of Rap signal in pre-TCR-mediated beta-selection checkpoint in alpha/beta T-cell development. *Blood*. 2008;112(12):4565-4573.
- Chu H, Awasthi A, White GCII, Chrzanoska-Wodnicka M, Malarkannan S. Rap1b regulates B cell development, homing, and T cell-dependent humoral immunity. *J Immunol*. 2008;181(5):3373-3383.
- Duchniewicz M, Zemojtel T, Kolanczyk M, Grossmann S, Scheele JS, Zwartkuis FJ. Rap1A-deficient T and B cells show impaired integrin-mediated cell adhesion. *Mol Cell Biol*. 2006;26(2):643-653.
- Chen Y, Yu M, Podd A, et al. A critical role of Rap1b in B-cell trafficking and marginal zone B-cell development. *Blood*. 2008;111(9):4627-4636.
- Dupuy AG, L'Hoste S, Cherfils J, Camonis J, Gaudriault G, de Gunzburg J. Novel Rap1 dominant-negative mutants interfere selectively with C3G and Epac. *Oncogene*. 2005;24(28):4509-4520.
- Hobeika E, Thiemann S, Storch B, et al. Testing gene function early in the B cell lineage in mb1-cre mice. *Proc Natl Acad Sci U S A*. 2006;103(37):13789-13794.
- Hamazaki Y, Fujita H, Kobayashi T, et al. Medullary thymic epithelial cells expressing Aire represent a unique lineage derived from cells expressing claudin. *Nat Immunol*. 2007;8(3):304-311.
- Fleming HE, Paige CJ. Pre-B cell receptor signaling mediates selective response to IL-7 at the pro-B to pre-B cell transition via an ERK/MAP kinase-dependent pathway. *Immunity*. 2001;15(4):521-531.
- Milne CD, Paige CJ. IL-7: a key regulator of B lymphopoiesis. *Semin Immunol*. 2006;18(1):20-30.
- Morrow MA, Lee G, Gillis S, Yancopoulos GD, Alt FW. Interleukin-7 induces N-myc and c-myc expression in normal precursor B lymphocytes. *Genes Dev*. 1992;6(1):61-70.
- Seet CS, Brumbaugh RL, Kee BL. Early B cell factor promotes B lymphopoiesis with reduced interleukin 7 responsiveness in the absence of E2A. *J Exp Med*. 2004;199(12):1689-1700.
- Kee BL, Rivera RR, Murre C. Id3 inhibits B lymphocyte progenitor growth and survival in response to TGF-beta. *Nat Immunol*. 2001;2(3):242-247.
- Sasagawa S, Ozaki Y, Fujita K, Kuroda S. Prediction and validation of the distinct dynamics of transient and sustained ERK activation. *Nat Cell Biol*. 2005;7(4):365-373.
- He Y-W, Malek TR. Interleukin-7 receptor α is essential for the development of $\gamma\delta^+$ T cells, but not natural killer cells. *J Exp Med*. 1996;184(1):289-293.
- Ye SK, Maki K, Kitamura T, et al. Induction of germline transcription in the TCR γ locus by Stat5: implications for accessibility control by the IL-7 receptor. *Immunity*. 1999;11(2):213-23.
- Bain G, Maandag EC, Izon DJ, et al. E2A proteins are required for proper B cell development and initiation of immunoglobulin gene rearrangements. *Cell*. 1994;79(5):885-892.
- Murre C. Helix-loop-helix proteins and lymphocyte development. *Nat Immunol*. 2005;6(11):1079-1086.
- Kwon K, Hutter C, Sun Q, et al. Instructive role of the transcription factor E2A in early B lymphopoiesis and germinal center B cell development. *Immunity*. 2008;28(6):751-762.
- Quong MW, Martensson A, Langerak AW, Rivera RR, Nemazee D, Murre C. Receptor editing and marginal zone B cell development are regulated by the helix-loop-helix protein, E2A. *J Exp Med*. 2004;199(8):1101-1112.
- Carvalho TL, Mota-Santos T, Cumano A, Demengeot J, Vieira P. Arrested B lymphopoiesis and persistence of activated B cells in adult interleukin 7(-/-) mice. *J Exp Med*. 2001;194(8):1141-1150.
- Christian SL, Lee RL, McLeod SJ, et al. Activation of the Rap GTPases in B lymphocytes modulates B cell antigen receptor-induced activation of Akt but has no effect on MAPK activation. *J Biol Chem*. 2003;278(43):41756-41767.
- Lin KB, Freeman SA, Zabetian S, et al. The rap GTPases regulate B cell morphology, immunosynapse formation, and signaling by particulate B cell receptor ligands. *Immunity*. 2008;28(1):75-87.
- Iritani BM, Delrow J, Grandori C, et al. Modulation of T-lymphocyte development, growth and cell size by the Myc antagonist and transcriptional repressor Mad1. *EMBO J*. 2002;21(18):4820-4830.
- Engel I, Johns C, Bain G, Rivera RR, Murre C. Early T lymphocyte development is regulated by modulation of E2A protein activity. *J Exp Med*. 2001;194(6):733-745.
- Wang S-F, Aoki M, Shinozuka Y et al. Development of Notch-dependent T-cell leukemia by deregulated Rap1 signaling. *Blood*. 2008;111(5):2878-2886.